Basic Investigations

A Proteomical Study on the Radiosensitized Target Molecules of Fuzheng Zengxiao Formula (扶正增效方) in Pulmonary Adenocarcinoma Nude Mice Model

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Objective: To investigate the radiosensitized target of Fuzheng Zengxiao Formula (扶正增效方).
Methods: The pulmonary adenocarcinoma (PAa) nude mice of tumor transplantation model were prepared and divided into four groups: Group I (blank control group, *n*=10), Group II (simple radiotherapy group, *n*=10), Group III (radiotherapy plus Fuzheng Zengxiao Formula, *n*=10) and Group IV (radiotherapy plus metronidazole, *n*=10). Radiation of X-rays was given to the tumors in Group I, II and III when they were averagely about 1 centimetre in diameter. 23 hours later, the tumors were taken, the total proteins were extracted, and the protein contents were determined. The proteins were isolated with two dimensional gel electrophoresis, and the differentially expressed proteins were analyzed with mass spectrometry and identified by protein database.
Results: Six significant proteins, including apolipoprotein E, ceratin75, S100A9, cyclophilin A, S100A10 and hemoglobin, were determined. Compared with Group I, apolipoprotein E and ceratin75 highly expressed in the Group II; compared with Group II, S100A9, cyclophilin A and hemoglobin had high expression in the Group III; compared with Group II, S100A9, cyclophilin A, S100A10 and hemoglobin had high expression in the Group IV; compared with Group IV, S100A9 and S100A10 had low expression and hemoglobin had high expression in Group III.
Conclusion: The radiosensitization of Fuzheng Zengxiao Formula is related with the improvement of hypoxia state; and possibly S100A9 and cyclophilin A are the target proteins of Fuzheng Zengxiao Formula in radiosensitization.

Keywords: *radiosensitization; Fuzheng Zengxiao Formula; proteomics*

The authors' previous clinical observation should that the effective rate reached to 69.4% in non-small cell pulmonary cancer patients treated by radiotherapy plus Fuzheng Zengxiao Formula (扶正增效方) at stage III and IV, and the survival rates of 1, 2, 3 years were respectively 79.4%, 49.4% and 23.3%.[1] The animal experiment has demonstrated that the radiosensatization ratio of the TCM formula is 1.26.[2] The study with gene chip indicates that the radiosensitization of Fuzheng Zengxiao Formula for pulmonary adenocarcinoma (PAa) is regulation of cell cycles, apoptosis, signal transduction and metabolism, etc.[3] In order to comprehensively recognize the mechanism of Fuzheng Zengxiao Formula at protein level, comparative proteomics was adopted in this study.

METHODS

Medicines

Fuzheng Zengxiao Formula is composed of Sheng Huang Qi (Raw Radix Astragali), Shi Hu (Herba Dendrobii), Sha Shen (Radix Adenophorae Strictae), Yin Hua (Flos Lonicerae), Hong Hua (Flos Carthami), and Su Mu (Lignum Sappan), etc., which should be decocted twice, with the decoctions mixed and concentrated into 2.2 g crude drugs/mL, and then disinfected by boiling method.

Animals

Forty male BALB/C-nu/nu nude mice, aged 6 weeks, were purchased from the Institute of Experimental Animals, Chinese Academy of Medical Sciences (SCSK 京2005-0013), and raised in SPF condition.

PAa Cell Line

The low transfer human pulmonary adenocarcinoma was purchased from the Pathological Section of Medical Department, Peking University.

Instruments and Reagents

Calf serum and DEPC were the products of Sigma, USA. IPGphor isoelectric focusing electrophoresis apparatus, video-scanner, Protean Ⅱ vertical electrophoresis apparatus, HD MS mass spectrometer, solid-phase pH gradient xerogel trip (PH3-10, linear, 70 mm × 3 mm × 0.5 mm, IPGs), and the related 2-DE reagents, lysate, balanced solution were purchased from Bio-Rad Co. USA.

Preparation of Human Pulmonary Adenocarcinoma Nude Mice Model and the Treatment

The cells of the pulmonary adenocarcinoma at logarithmic growth phase were taken. After trypsinization with 0.1% trypsin containing 0.01% EDTA,

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they were regulated into 5 × 10^6/mL with RPMI1640 culture medium, and then 0.3 mL was injected into the subaxillary part of the anterior limb in the nude mice. The mice were randomly divided into Group I (blank group), Group II (simple radiotherapy), Group III (radiotherapy plus Fuzheng Zengxiao Formula) and Group IV (radiotherapy plus metronidazole), 10 mice in each group. From the innovation day, each nude mouse in Group III was intragastrically administrated with 0.5 mL（1.1 g crude drug）Fuzheng Zengxiao Formula every day, Group IV with 0.5 mL (10 mg) metronidazole solution, and the other two groups with equal volume of distilled water. When the tumor body was 1 cm in diameter (about 30 days later), 10 nude mice with no obvious ulcer on the tumor surface was selected from each group, and the tumor was radiated once with 10 Gy 12 MeV β electric ray except Group I.

Extraction of Proteins and the Content Determination

Twenty-three h after the radiation, the tumor body was taken and homogenized, and the protein components in cells were extracted with the three-step extraction method, i.e., extraction with water solution, solution containing Urea- CHAPS-DTT and different lysates containing sulfourea- SB3-10-TBP, and then the protein contents were determined with Bradford method.

Two Dimensional Gel Protein Electrophoresis (2-DE)

In reference to the literuature,[4] 300 μg sample proteins were taken from each group for 2-DE electrophoresis, and the gel was stained with Coomassie blue G-250 (Bio-Rad). The digital image was obtained by Imagescan scanner and analyzed with photoshop8.0 image software. This experiment was repeated thrice.

Mass Spectrography and Biological Information Analysis of the Differential Protein Dots

The differential protein dots with stable reduplication were selected from the above 3 experiments, and 24 differential protein dots were cut for substrate accessory laser desorption ionization flying time mass spectrography, which was done by the National Bio-Medical Analysis Center, China Academy of Military Medical Sciences, with a ReFlexⅢmass spectroscope (Bruker Company), obtaining a peptide mass fingerprinting (PMF), Mascot (http://www.matrixscience.co.uk) on-line searching protein series data bank database of SWISS-PORT and NCBInr. The retrieving condition: with Trypsinization, M-oxidation and iodoacetamide alkylation as the variable modification, the lapse cutting site was 1. The mass errors of both MS and MS/MS were 0.2 Da. Based on the determined protein information, the common data of Ucsc, TRANSFAC, NCBI, Gobet, etc. were retrieved and the biological information was analyzed.

RESULTS

Two Dimensional Electrophoretogram

The exacted proteins from Group I, II, III and IV were isolated with 2-DE and duplicated 3 times. The protein dots of the 3 times were 1218±45 in Group I, 1240±27 in Group II, 1397±32 in Group III and 1132±25 in Group IV. The common matching dots of Group III and II were 984 with a matching rate of 74.57% , and the protein dots with a variation more than two times were 235 (119 up-regulated and 116 down-regulated). The common matching dots of Group IV and II were 892 with a matching rate of 75.14%, and the protein dots with a variation more than two times were 221 (161 up-regulated and 60 down-regulated). The common matching dots of Group I and II were 961 with a matching rate of 78.16% , and the protein dots with a variation more than two times were 204 (151 up-regulated and 53 down-regulated). It is indicated that the isolated proteins were dependable.

Differential Dots of Cellular Protein Expression

By searching and statistical analysis of gel electrophoretograms with PDQuest software, comparison of the cellular protein profiles among the 4 groups showed that 6 dots had more obvious change in protein expression amount. These 6 obviously differential protein dots were taken for enzymolysis in the gel, and analyzed with ultrahigh-performance liquid chromatograpgy and high-accuracy mass spectrometry, obtaining the peptide mass fingerprintings of the 6 protein dots.

The Mass Spectrography and Bioinformatics Analyze for Differential Protein Dots

Based on the acquired mass-spectrum peaks and after confirmation with Mascot software, the possible protein information mainly includes S100A9 and S100A10 involved in inhibiting the activities of casein kinase I and II, cyclophilin involved in signal transduction and catalyzing folding, assemblage and transportation of proteins, apolipoprotein involved in immune modulation, hemoglobin carrying oxygen, and ceratin, etc (Table 1).

Table 1. The proteins expressed by different groups

Dot	No	Matching peptide segment	Maximum score	Theoretically molecular mass	Protein	Function
1	gi4557325	8	66	36246	apolipoprotein E	involved in lipid metabolism
2	gi29789317	9	87	59704	ceratin75	cellular constitution
3	gi6677837	6	60	13234	S100A9	cellular differentiation, and apoptosis, etc
4	gi509233	10	62	17870	cyclophilin A	protein chaperoning
5	gi229301	3	115	15653	hemoglobin	transporting oxygen
6	gi6677833	7	140	11179	S100 A10	cellular differentiation, and apoptosis, etc

DISCUSSION

Fuzheng Zengxiao Formula is a radiosensitizing recipe devised by Prof. ZHANG Dai-zhao for the toxic heat injuring yin and hypercoagulability in pulmonary cancer patients treated by radiotherapy. It is composed of Sheng Huang Qi (raw Radix Astragali), Shi Hu (Herba Dendrobii), Sha Shen (Radix Adenophorae Strictae), Yin Hua (Flos Lonicerae), Hong Hua (Flos Carthami) and Su Mu (Lignum Sappan), etc. The experimental study has proved the radiosensatization of Fuzheng Zengxiao Formula in immune modulation, apoptosis, repair of DNA, expressions of cancer gene and cancer suppressor gene, and gene mathylization, etc. However, most studies on radiosensatization stay at mono-factor correlativity studies, but the specific factor of radiosensitization has not found yet. Comparative proteomics is aimed at finding the differential protein profile between samples, exploring the course and essence of the cellular physiological and pathological states, and the cellular regulation mechanisms; and at the same time, obtaining the qualitative and functional analysis for some key proteins. Therefore, comparative proteomics is of important significance in early diagnosis, and in analysis of the course and treatment of diseases.

In the present study, with the comparative proteomics methods and by using the two dimensional electrophoresis and MAIDI-MS-MS, the protein differential expression profiles were investigated in the blank control group, the simple radiotherapy group, the radiotherapy plus Fuzheng Zengxiao Formula group, and the metronidazole plus radiotherapy group. The results showed that the 2-DE system had a good reproducibility, providing a basis for finding the differential expression proteins. Comparison and analysis of the protein expression profiles revealed that there were differences in protein expression in all the groups, indicating that both Fuzheng Zengxiao Formula and metronidazole exerted effects on protein expression after radiotherapy, and the radioactive ray also showed effects on protein profiles of the tumor body. Ten protein dots with obviously differential expression selected and analyzed, and the peptide mass fingerprints were obtained to be identified. Of them, 6 protein dots with satisfactory matching results were identified.

Compared with the blank control group, apolipoprotein E and ceratin 75 had high expression in the simple radiotherapy group. Apolipoprotein E is of obvious and rich polymorphism, and the polymorphism determines individual blood lipid level, which is closely related with the genesis and development of atherosclerosis. In addition, apolipoprotein E is also involved in activation of the enzymes of hydrolyzing fats, and in immune modulation and regeneration of the nervous tissues. Ceratin exists widely in hair, nails and cell membrane of the animals. The ceratin expression change after radiotherapy is possibly caused by radio-ray effects.

Compared with the simple radiotherapy group, high expressions of S100A9, cyclophilin A, hemoglobin and S100A10 were found in the protein profiles of the metronidazole plus radiotherapy group. S100A9 and S100A10 of calgranulin B are members of the calcium binding protein family. Different molecules of the S100 family have wide biological activities, they play important roles in cell proliferation and differentiation, muscular contraction, gene expression, secretion and cell apoptosis. Calgranulin B can inhibit the activities of casein kinase I and II, and it may show abnormal expression in pulmonary cancer tissue. It is reported that in radiotherapy of cervical carcinoma, calgranulin B has strong expression, and the expression rate is higher in the high sensitization group than that in the low sensitization group.[5] Hemoglobin is the oxygen molecule transportation carrier, and in the lung it combines with oxygen molecules to form oxyhemoglobin, which transports oxygen to the tissues and participates in the oxidation-reduction reaction in human body. Cyclophilin A (CyPA) is a growth factor of oxidation stress-induced secretion. At the oxidation stress, CyPA secretes from the vascular smooth muscle cells to the outside of cells, mediating the active oxygen to activate the extracellular signal regulation proteins. A study indicates that CyPA is involved in the genesis and development of many cancers (including pulmonary cancer).[6] The results of the present study suggests that the possible mechanism of the radio sensitization in the metronidazole plus radiotherapy group is related with the inhibition of proliferation, differentiation, gene expression and apoptosis of the tumor cells, and also with the improvement of oxygen supply in tumor tissue.

Compared with the simple radiotherapy group, high expressions of S100A9, CyPA and hemoglobin were found in the protein profiles of the Fuzheng Zhenxiao Formula plus radiotherapy group with significant up-regulation. The authors' previous study indicated that the mechanism of radiosensatization of the Fuzheng Zhenxiao Formula was possibly related with the improvement of anaerobic condition in the cancer tissue. But hemoglobin is produced by bone marrow hematopoietic cells, not by the tumor tissues. The hemoglobin found in differential proteins is possibly caused by transportation of oxygen into the tumor tissue, so it is not the target of radiosensatization of the Fuzheng Zhenxiao Formula. It is indicated that Fuzheng Zhenxiao Formula plays a role in cellular proliferation, differentiation, apoptosis and signal transduction, etc., which are identical with the results of the gene expression profiles.

Compared with the metronidazole plus radiotherapy group, low expression of the members of S100 calcium binding protein family and high expression of

hemoglobin were found in the Fuzheng Zhenxiao Formula plus radiotherapy group, indicating that their radiosensitization mechanisms are not completely the same, and the Fu Zheng Zhen Xiao Formula has more obvious action in improving hypoxia. Nevertheless, some of the differential dots can not be detected from the differential profiles, which needs to be further studied.

REFERENCES

1. Huang JC, Zhang DZ. Observation on the long-term therapeutic effects of Fuzheng Zhenxiao Formula combined with radiotherapy on pulmonary cancer. China Med J (Chin) 2000; 35: 48.
2. Huang JC, Zhang DZ. Study on radiosensitization of Fuzheng Zhenxiao Formula on pulmonary adenocarcinoma cells. China J Radio-oncol (Chin) 2000; 9: 135-136.
3. Huang J, Huang JC. Analysis on gene expression profiles of radiosensitization of Fuzheng Zhenxiao Formula on pulmonary adenocarcinoma cells. J Chin-Jpn Friendship Hospital (Chin) 2006; 20: 348-351.
4. Sun HL, Liu J, Ding F, Wang X, Liu M, Gu X. Investigation of differentially expressed proteins in gastrocnemius muscle during denervation-reinnervation. J Muscle Res Cell Motil 2006; 27: 241-250.
5. Zhu H, Zheng L, Tu QS, Xiao ZQ. Expression and significance of S100A9, Galectin7 in cervical carcinoma with different sensitivities at same time of radio-chemotherapy. China J Clin Oncol (Chin) 2008; 35: 277-280.
6. Feng YM, Wu WD, Yan Z, Yao Wu, Wu YM. Relation between the cyclophilin expression and clinical pathological haracteristics in non-small cell pulmonary cancer. Acad J Second Milit Med University (Chin) 2008; 29: 146-149.

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